

## CLINICAL NEPHROLOGY – EPIDEMIOLOGY – CLINICAL TRIALS

## Tuberculin skin testing underestimates a high prevalence of latent tuberculosis infection in hemodialysis patients

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**Tuberculin skin testing underestimates a high prevalence of latent tuberculosis infection in hemodialysis patients.**

**Background.** Identification of latent *Mycobacterium tuberculosis* infection in hemodialysis patients is hampered by reduced sensitivity of the established tuberculin skin test. We investigated whether in vitro quantitation of purified protein derivative (PPD)-specific T cells using a rapid 6-hour assay may represent an alternative approach for detecting latent infection.

**Methods.** One hundred and twenty-seven hemodialysis patients and 218 control patients (blood donors, health care workers, and control patients) were analyzed. Specific T cells toward PPD and early secretory antigenic target-6 (ESAT-6), a protein expressed in *Mycobacterium tuberculosis* but absent from *M. bovis* bacillus Calmette-Guerin (BCG) vaccine strains, were flow cytometrically quantified from whole blood, and results were compared with skin testing.

**Results.** Compared to blood donors, a high proportion of both health care workers (48.6%) and hemodialysis patients (53.5%) had PPD-specific Th1-type CD4 T-cell reactivity with similar median frequencies of PPD-specific T cells (0.17%; 0.06–3.75% vs. 0.26%; 0.06–4.12%, respectively). In contrast, skin test reactivity was significantly reduced in hemodialysis patients. Whereas 85.7% of control patients with PPD reactivity in vitro were skin test-positive, the respective percentage among hemodialysis patients was 51.4% ( $P = 0.007$ ). Among individuals with PPD reactivity in vitro, ~50% had T cells specific for ESAT-6.

**Conclusion.** Unlike the skin test, measurement of PPD reactivity by in vitro quantitation of PPD-specific T cells was unaffected by uremia-associated immunosuppression. This whole-blood assay may thus be a valuable alternative to skin testing, and detection of ESAT-6-specific T cells could moreover allow distinction of latent *M. tuberculosis* infection from BCG-induced reactivity to PPD. The assay is well suited for clinical use and may facilitate targeting of preventative therapy in high-risk individuals.

**Key words:** latent tuberculosis infection, T-cell response, flow cytometry, tuberculin skin test, hemodialysis, immunodeficiency.

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Tuberculosis is one of the leading infectious diseases in man that accounts for around eight million cases and nearly three million deaths annually. The disease is caused by infection with *Mycobacterium tuberculosis* and represents an important cause of morbidity and mortality, especially among immunocompromised individuals [1].

Because of systemic immunosuppression, individuals such as human immunodeficiency virus (HIV)-infected patients, transplant recipients, and hemodialysis patients with *M. tuberculosis* infection are at increased risk of progression to active disease. Chronic renal failure is associated with a state of uremic immunodeficiency that is clinically characterized by a high incidence of infectious complications and attendant mortality [2], by an impaired response to vaccinations, and by reduced delayed-type hypersensitivity reactions [3–6]. The molecular basis for the altered in vivo immune response is associated with impaired activation of T cells [7, 8]. The defect itself resides in antigen-presenting cells [9], as it may be compensated in vitro by the application of costimulatory signals of the B7/CD28-pathway [10, 11]. Apart from an altered costimulatory capacity, we and others found a bias of T-helper cell responses (Th) toward Th1 [12], and an increased production of proinflammatory cytokines in monocytes from patients undergoing chronic intermittent hemodialysis [13–15]. Interestingly, as with decreased costimulatory capacity, the extent of expression of proinflammatory cytokines also correlates with the severity of clinical immunodeficiency [14].

Prevention of the development of active tuberculosis critically depends on sensitive diagnostic tests to identify latently infected individuals. A latent infection is typically characterized by a relatively strong PPD-specific immune response in the absence of detectable bacterial load. The tuberculin skin test is currently the only widely used diagnostic method for the identification of an *M. tuberculosis* infection in individuals without disease [16]. A major disadvantage of the tuberculin skin test is the impaired sensitivity in high-risk groups of immunocompromised patients. Based on sensitivity, specificity, and on the

prevalence of tuberculosis, different cutpoints have been suggested for defining a positive skin-test reaction, and for recommendations for prophylaxis [17, 18]. A cutpoint of  $\geq 15$  mm applies to immunocompetent individuals. In contrast, for individuals with mildly impaired immunity such as hemodialysis patients, a lower threshold of  $\geq 10$  mm is suggested. Nevertheless, depending on the extent of immunosuppression, the actual number of latently infected individuals may be considerably underestimated [5, 6].

At present, there is no standard diagnostic test to diagnose latent tuberculosis infections in anergic patients. This would be of considerable clinical importance, as this could aid in identifying latently infected patients, and in targeting appropriate preventative therapy before transplantation. Recently, a number of assays systems have been described that allow the rapid analysis of pathogen-specific T-cell immunity in vitro [19–21]. This study was performed to characterize the T-cell response toward purified protein derivative (PPD) from *M. tuberculosis* and to analyze whether its direct quantitation from whole blood could represent an alternative approach to the skin test in anergic and/or immunocompromised patients. Moreover, reactivity toward the recombinant early secretory antigenic target 6 (ESAT-6) protein, which is absent from all strains of the *M. bovis* bacillus Calmette-Guerin (BCG) vaccine, was analyzed to distinguish true *M. tuberculosis*-specific immune responses from vaccination-induced responses. Hemodialysis patients with uremia-associated immunodeficiency were chosen as a group of individuals with increased risk for progression to active disease. Immunocompetent medical staff as well as healthy blood donors were studied as controls for individuals with similar or low risk of exposure, respectively.

## METHODS

### Subjects

The study was conducted among 127 hemodialysis patients ( $61.2 \pm 15.2$  years) and 218 immunocompetent control individuals ( $44.0 \pm 14.8$  years) of Caucasian origin. Control individuals consisted of health care workers ( $N = 107$ ), immunocompetent patients ( $N = 59$ ) who were admitted to the Medical Departments IV and V (University of the Saarland, Germany), and who underwent a routine screening for PPD-specific T-cell reactivity between December 2001 and December 2002, and healthy blood donors ( $N = 52$ ). Study participants had no signs or symptoms of active tuberculosis. Subclinical infections were also unlikely because patients were seen regularly, and none of the individuals have presented with active disease during the 8-month period since the end of the study. Exclusion criteria for control individuals were the presence of chronic diseases associated with nonspe-

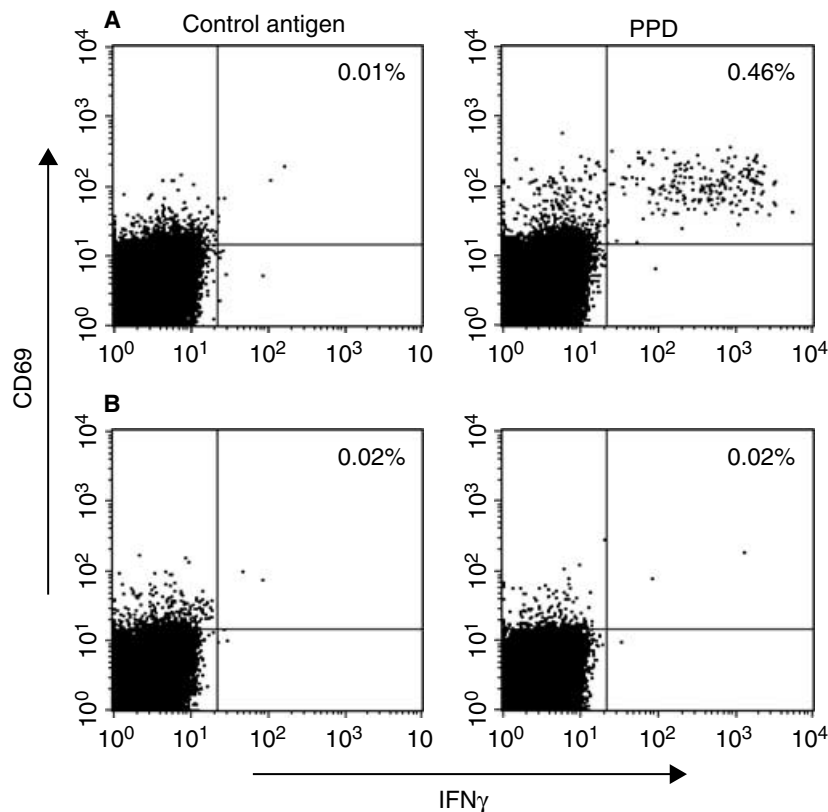
cific immunosuppression (diabetes, liver or renal diseases, HIV infection, malnutrition, or malignant diseases) and the use of immunosuppressive medication. BCG (bacillus Calmette-Guerin) vaccination status was not consistently available for all study participants. The etiology of end-stage renal failure in hemodialysis patients was due to glomerulonephritis ( $N = 28$ ), pyelonephritis ( $N = 7$ ), diabetic nephropathy ( $N = 39$ ), polycystic kidney disease ( $N = 11$ ), vascular nephropathies ( $N = 10$ ), systemic vasculitis ( $N = 2$ ), interstitial diseases ( $N = 3$ ), others ( $N = 12$ ), and unknown ( $N = 15$ ). Blood from patients was drawn before the start of the hemodialysis session. The study was approved by the local ethics committee, and all individuals gave informed consent.

### Stimulation of PPD-specific CD4 T cells within whole blood

Stimulation of PPD-specific CD4 T cells was performed from heparinized whole blood essentially as described for the detection of virus-specific T cells [20, 22–24]. As a stimulus, titrated amounts of PPD (222 IU/mL, Tuberkulin GT-1000; Chiron Behring, Marburg, Germany) or 10  $\mu$ g/mL recombinant ESAT-6 protein (a kind gift of A. Whelan, TB Research Group, Veterinary Laboratories Agency, Weybridge, UK) were used in the presence of 1  $\mu$ g/mL  $\alpha$ CD28 and  $\alpha$ CD49d (clones L293 and 9F10, BD, Heidelberg, Germany), respectively. As negative controls, cells were stimulated with the diluent that was used to dissolve PPD (Chiron Behring). Cells were incubated in polypropylene tubes at 37°C at 6% CO<sub>2</sub> for a total of 6 hours. During the last 4 hours, 10  $\mu$ g/mL of Brefeldin A (Sigma, Deisenhofen, Germany) was added to block extracellular secretion of cytokines. Thereafter, the blood was treated with 2 mmol/L EDTA for 15 minutes. Subsequently, erythrocytes were lysed, and leukocytes were fixed for 10 minutes using BD lysing solution (BD). Cells were washed once with fluorescence-activated cell sorter (FACS) buffer [phosphate-buffered saline (PBS), 5% filtered fetal calf serum (FCS), 0.5% bovine serum albumin (BSA), 0.07% NaN<sub>3</sub>] and processed for flow cytometric analysis.

### Determination of the frequency and characterization of PPD- and ESAT-6-specific T cells

Fixed leukocytes were permeabilized with 2 mL FACS buffer containing 0.1% saponin (Sigma) for 10 minutes at room temperature. Thereafter, they were triple- or quadruple-stained for 30 minutes at room temperature in the dark using saturating conditions of the following antibodies: anti-CD4 (clones SK3), anti-IFN $\gamma$  (clone 4S.B3), anti-TNF $\alpha$  (clone Mab11), anti-CD69 (clone L78). In addition, the cell surface molecules CD45RO and CD27 were analyzed on cytokine-positive cells using antibody clones UCHL1 and L128, respectively (all antibodies



**Fig. 1. Flow-cytometric quantitation of purified protein derivative (PPD)-specific CD4 T cells. Representative dot plots of individuals with (A) or without (B) detectable specific T-cell reactivity toward PPD.** Whole blood was stimulated with control antigen (diluent) or PPD and specifically induced interferon  $\gamma$  (IFN $\gamma$ ) induction in CD4 T cells was analyzed using flow cytometry. Numbers indicate the percentage of specifically stimulated CD4 T cells.

from BD). Thereafter, cells were washed once with 3 mL of FACS buffer and fixed with 1% paraformaldehyde. At least 15,000 CD4 T cells were analyzed on a FACS Calibur (BD) using Cellquest-Pro 4.0.2. The percentage of specific T cells was calculated by subtraction of the frequency obtained by the respective control stimulation. The lower limit of detection was 0.05%.

#### Determination of PPD reactivity in vivo

Reactivity toward PPD in vivo was determined using the skin test according to Mendel-Mantoux in accordance with standard national German guidelines (Tuberkulin GT-10; Chiron Behring). The test was carried out according to the manufacturer's instructions by the intracutaneous inoculation of 10U of PPD into the volar surface of the forearm. The resulting induration was determined by three qualified persons (U.S., P.C., and U.M.) 48 to 72 hours thereafter and compared with the reactivity toward the diluent. An induration of 5 mm was chosen as the internationally recognized cut-off for Mendel-Mantoux skin-test reactivity. Exclusion criteria for the performance of the skin test were a known history of tuberculosis infection, skin disorders, intercurrent pyrexial illness, or the lack of informed consent for the skin test.

#### Statistical analysis

Statistical analysis was performed using Prism V3.03 software (Graphpad, San Diego, CA, USA). Significant

differences were determined using the Mann-Whitney test. Fisher exact test and  $\chi^2$  test was used to analyze differences between two or more groups of individuals with respect to the prevalence of PPD reactivity or skin-test responses. Correlations were calculated according to Spearman.

## RESULTS

#### Similar prevalence and frequency distribution of PPD-specific CD4 T cells in immunocompetent health care workers and hemodialysis patients

PPD-specific CD4 T cells were flow cytometrically quantified from whole blood after stimulation with PPD in vitro. The frequency of specific cells was determined as the percentage of CD69 and interferon (IFN) $\gamma$ -positive CD4 T cells. Characteristic examples of individuals reactive and not reactive toward PPD are shown in Figure 1. Whereas 0.46% of CD4 T cells were activated to produce IFN $\gamma$  after stimulation with PPD (Fig. 1A), no such induction was observed in the individual shown in Figure 1B. Within each individual, the diluent failed to induce any relevant cytokine production. Nevertheless, the frequency of PPD-specific CD4 T cells was calculated by subtraction of the frequency obtained by the respective control stimulation.

This flow cytometric assay was used to analyze the frequencies of PPD-specific CD4 T cells in 127 hemodialysis

**Table 1.** Hemodialysis patients and immunocompetent health care workers do not differ in the prevalence of PPD reactivity in vitro

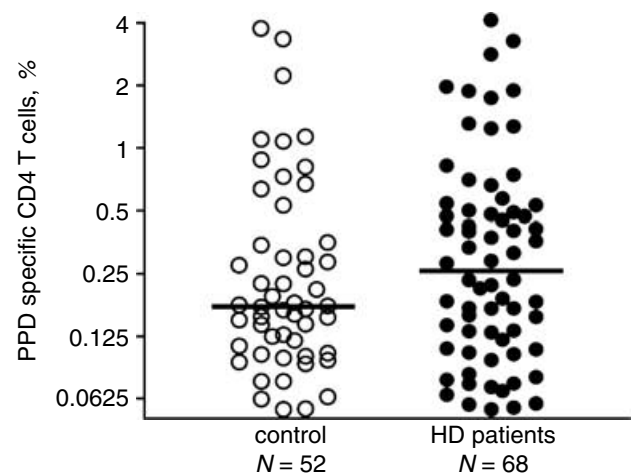
	Blood donors <i>N</i> = 52	Health care workers <i>N</i> = 107	Hemodialysis patients <i>N</i> = 127
PPD-specific CD4 T cells >0.05%	16 (30.8%)	52 (48.6%)	68 (53.5%)

PPD, purified protein derivative. Shown are the numbers and percentages of individuals with PPD-specific CD4 T-cell reactivity above detection limit of 0.05%. There was a significant difference between blood donors and health care workers or hemodialysis patients ( $P = 0.02$ ).

patients and 159 immunocompetent individuals, respectively. To account for the higher risk of hemodialysis patients for regular exposure to infected patients, immunocompetent individuals were subdivided into a group of blood donors with presumptive low-risk exposure, and a cohort of health care workers at similarly increased risk for exposure as hemodialysis patients. Among blood donors, 30.7% of individuals (16/52) showed PPD-specific CD4 T cells above the detection limit of 0.05% (Table 1). In line with an increased risk for exposure, this percentage was higher in both health care workers and hemodialysis patients ( $P = 0.02$ , Table 1). Interestingly, however, despite uremic immunodeficiency, there was no difference in the prevalence of PPD-specific T-cell reactivity between health care workers and hemodialysis patients (48.6% and 53.5%, respectively;  $P = 0.51$ ) (Table 1). Although there was a considerable interindividual variation in the frequency of PPD-specific CD4 T cells among PPD-positive individuals that ranged from 0.06 to up to 4.12% (Fig. 2), median frequencies did not differ between healthy individuals (0.17%, range 0.06 to 3.75%), and hemodialysis patients (0.26%, range 0.06 to 4.12%;  $P = 0.28$ ; Fig. 2). Thus, PPD-specific T cells can be quantified directly from whole blood, and the prevalence of PPD reactivity is lower in blood donors compared with the higher risk groups of hemodialysis patients and health care workers. Interestingly, despite uremic immunodeficiency, neither the prevalence nor the frequency of distribution differed between hemodialysis patients and immunocompetent health care workers.

#### PPD-specific T cells predominantly consist of both immature and mature memory cells of Th1 phenotype

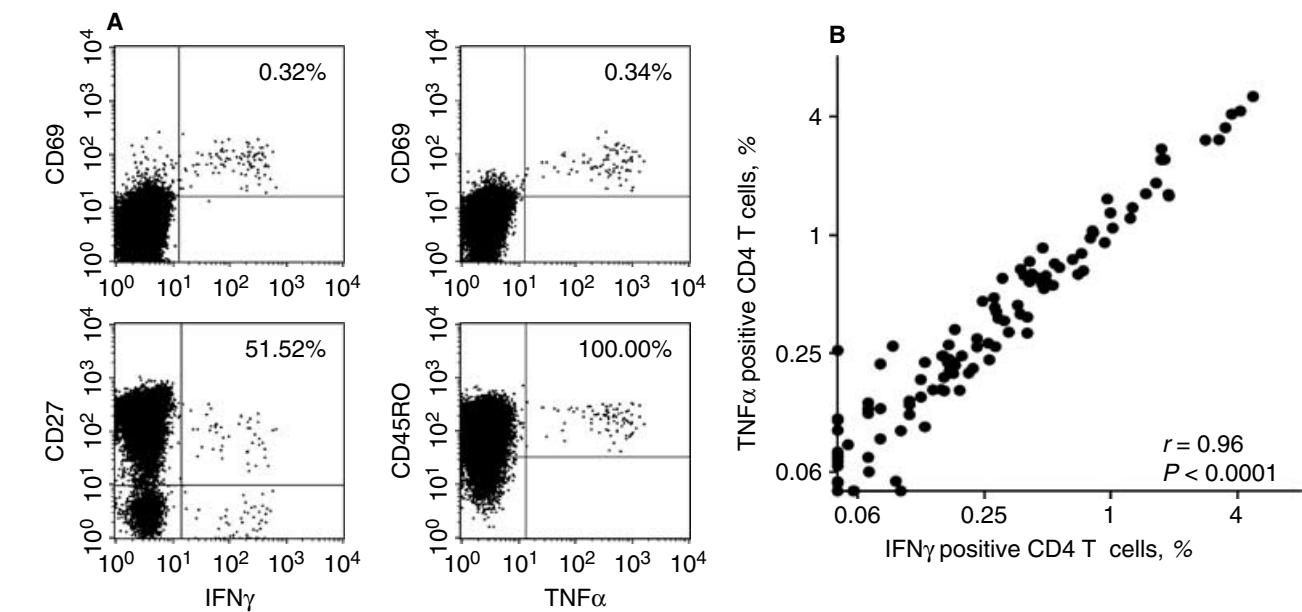
Phenotypical analysis was carried out to characterize the differentiation status and the cytokine profile of antigen-specific T cells. PPD-reactive T cells were shown to produce IFN $\gamma$  upon specific stimulation (Fig. 1). This cytokine induction was restricted to the CD4 T-cell subset (data not shown). The phenotype and maturation status of PPD-specific CD4 T cells was further characterized by the analysis of various other cytokines and cell surface markers (Fig. 3, Table 2). The induction of IFN $\gamma$  is in-



**Fig. 2. Heterogeneous frequencies of purified protein derivative (PPD)-specific CD4 T cells in immunocompetent individuals ( $N = 52$ , control patients) and hemodialysis patients ( $N = 68$ , HD patients) with PPD-specific T-cell reactivity.** Neither median T-cell frequencies nor the frequency distribution differed between control individuals (median 0.17%, range from >0.05% to 3.75%) and hemodialysis patients (median 0.26%, range from >0.05% to 4.12%;  $P = 0.28$ ).

dicative of a Th1 phenotype. In line with this evidence, a similar percentage of CD4 T cells was induced to produce the Th1 cytokine TNF $\alpha$  (Fig. 3A), and there was a strong correlation between the expression of both cytokines ( $r = 0.96$ ,  $P < 0.0001$ ; Fig. 3). In contrast, levels of Th2 cytokines such as interleukin (IL)4 and IL5 were in general below detection limit after stimulation with PPD ( $N = 13$ ; data not shown). The only exceptions were the hemodialysis patients with the highest frequencies of PPD-reactive IFN $\gamma$  and tumor necrosis factor (TNF) $\alpha$ -positive T cells (4.12% and 4.24%, respectively), who concomitantly had 0.21% of IL4-positive cells.

The majority of PPD-specific T cells showed a memory phenotype characterized by the expression of CD45RO (e.g.,  $90.88\% \pm 8.11\%$  of PPD-reactive CD4 T cells in hemodialysis patients) (Table 2). The analysis of CD27 served as a marker to determine the differentiation status of specific T cells. Interestingly, among PPD-specific T cells, there were similar percentages of CD27 positive and negative cells, indicating the existence of both recently activated and terminally differentiated T cells, respectively (CD27 positive:  $54.20 \pm 18.97\%$  in hemodialysis patients) (Table 2). There were no differences in the phenotype of PPD-specific T cells between hemodialysis patients and control patients (Table 2). A representative example, where all PPD-specific T cells were positive for CD45RO and 51.52% of the respective T cells were positive for CD27 is shown in Fig. 3A. Taken together, PPD-specific CD4 T cells predominantly comprise both recently activated as well as terminally differentiated memory cells of a Th1 phenotype.



**Fig. 3. Phenotypic characterization of purified protein derivative (PPD)-specific CD4 T cells.** Dot plots of the flow-cytometric analysis of CD45RO and CD27 expression among cytokine-positive CD4 T cells after stimulation with PPD (A). Numbers denote the frequencies of activated (CD69-positive) interferon  $\gamma$  (IFN $\gamma$ ) or tumor necrosis factor $\alpha$  (TNF $\alpha$ )-positive T cells among all CD4 T cells (*upper panel*), or the percentage of CD27- or CD45RO-positive T cells among cytokine-positive cells (*lower panel*). A significant correlation exists between the percentage of PPD-specific CD4 T cells producing IFN $\gamma$  or TNF $\alpha$ , respectively ( $r = 0.96$ ,  $P < 0.0001$ ;  $N = 134$ ) (B).

**Table 2.** PPD-specific CD4 T cells comprise both recently activated as well as mature memory Th1 cells

Hemodialysis patients	Percentage among cytokine-positive T cells	N
Phenotype		
CD27 positive	54.20 $\pm$ 18.97%	43
CD45RO positive	90.88 $\pm$ 8.11%	51
Controls		
Phenotype	Percentage among cytokine-positive T cells	N
CD27 positive	59.90 $\pm$ 14.90%	32
CD45RO positive	87.73 $\pm$ 11.37%	28

PPD, purified protein derivative. Numbers indicate the percentage of cells positive for the expression of CD45RO and CD27 among cytokine-positive CD4 T cells stimulated with PPD. There was no difference in the phenotype of PPD-specific CD4 T cells between immunocompetent individuals and hemodialysis patients.

**Lower percentage of skin test–positive individuals among hemodialysis patients**

In order to compare the in vitro reactivity toward PPD with the reactivity in vivo, a standard skin test according to Mendel-Mantoux was carried out in both immunocompetent individuals and hemodialysis patients. Immunocompetent individuals comprised both health care workers ( $N = 23$ ) and immunocompetent nonuremic patients ( $N = 33$ ) (Table 3). As expected, among individuals with PPD-specific T-cell frequencies below 0.05%, the majority (20/21 control patients and 29/29 hemodialysis patients) had an induration below 5 mm. Conversely, a positive skin test was found in 91.7% of health care workers and 82.6% of control patients with PPD reactivity above 0.05% (Table 3). In large contrast, how-

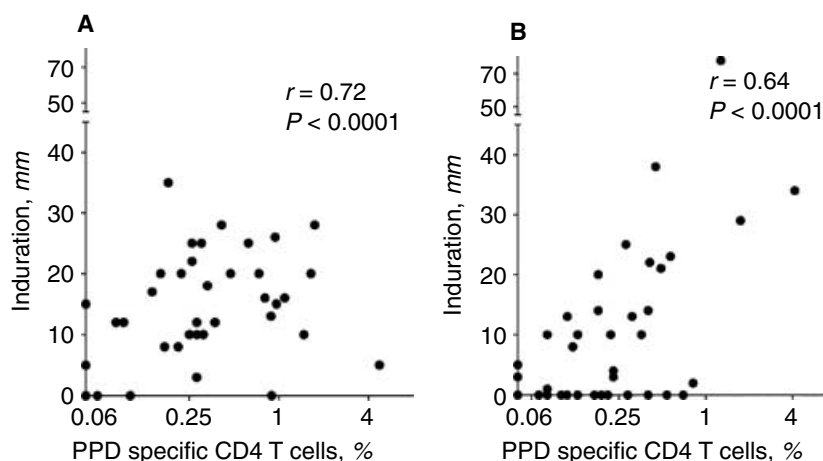
**Table 3.** Lower sensitivity of the skin test in hemodialysis patients compared with immunocompetent control patients

	Health care workers N = 12	Control patients N = 23	Hemodialysis patients N = 35
PPD-specific T cells >0.05%			
Skin-test >5 mm	11 (91.7%)	19 (82.6%)	18 (51.4%)

PPD, purified protein derivative. Shown is the percentage of individuals with an induration >5mm among individuals with PPD-specific T-cell frequencies above 0.05%. Median skin-test induration was 15 mm (range 0 to 35 mm) for control patients (health care workers and control patients), and 8 mm (range 0 to 78 mm) for hemodialysis patients. Twenty out of 21 immunocompetent control patients and 29/29 hemodialysis patients with PPD reactivity below 0.05% had an induration smaller than 5 mm. There was no difference in sensitivity between health care workers and immunocompetent control patients. $P = 0.007$ .

ever, a significantly lower percentage of positive skin tests was found among PPD-positive hemodialysis patients (51.4%,  $P = 0.007$ ) (Table 3).

Interestingly, a significant correlation was observed between the frequency of PPD-reactive CD4 T cells and the respective induration in the skin test, although the correlation was less tight in hemodialysis patients (immunocompetent controls:  $r = 0.72$ ,  $P < 0.0001$ ; hemodialysis patients:  $r = 0.64$ ,  $P < 0.0001$ ) (Fig. 4). Together, this suggests that the flow cytometric quantitation of PPD-specific CD4 T cells may represent a sensitive assay to detect PPD reactivity ex vivo. Moreover, in the face of similar prevalences of PPD reactivity (Fig. 2) and a decreased sensitivity of the skin test in hemodialysis patients compared with immunocompetent control patients (Table 3 and [5, 6]), the flow cytometric assay may be of



**Fig. 4. Correlation between induration and purified protein derivative (PPD)-specific T-cell frequencies.** A significant correlation exists between the frequency of PPD-specific CD4 T cells and the induration in skin test in control individuals (A) (health care workers and immunocompetent control patients,  $r = 0.72$ ,  $P < 0.0001$ ,  $N = 56$ ) and hemodialysis patients ( $r = 0.64$ ,  $P < 0.0001$ ,  $N = 63$ ) (B).

particular value for the sensitive detection of PPD reactivity in immunocompromised individuals.

#### Reactivity toward ESAT-6 allows the identification of a latent infection among PPD-positive individuals

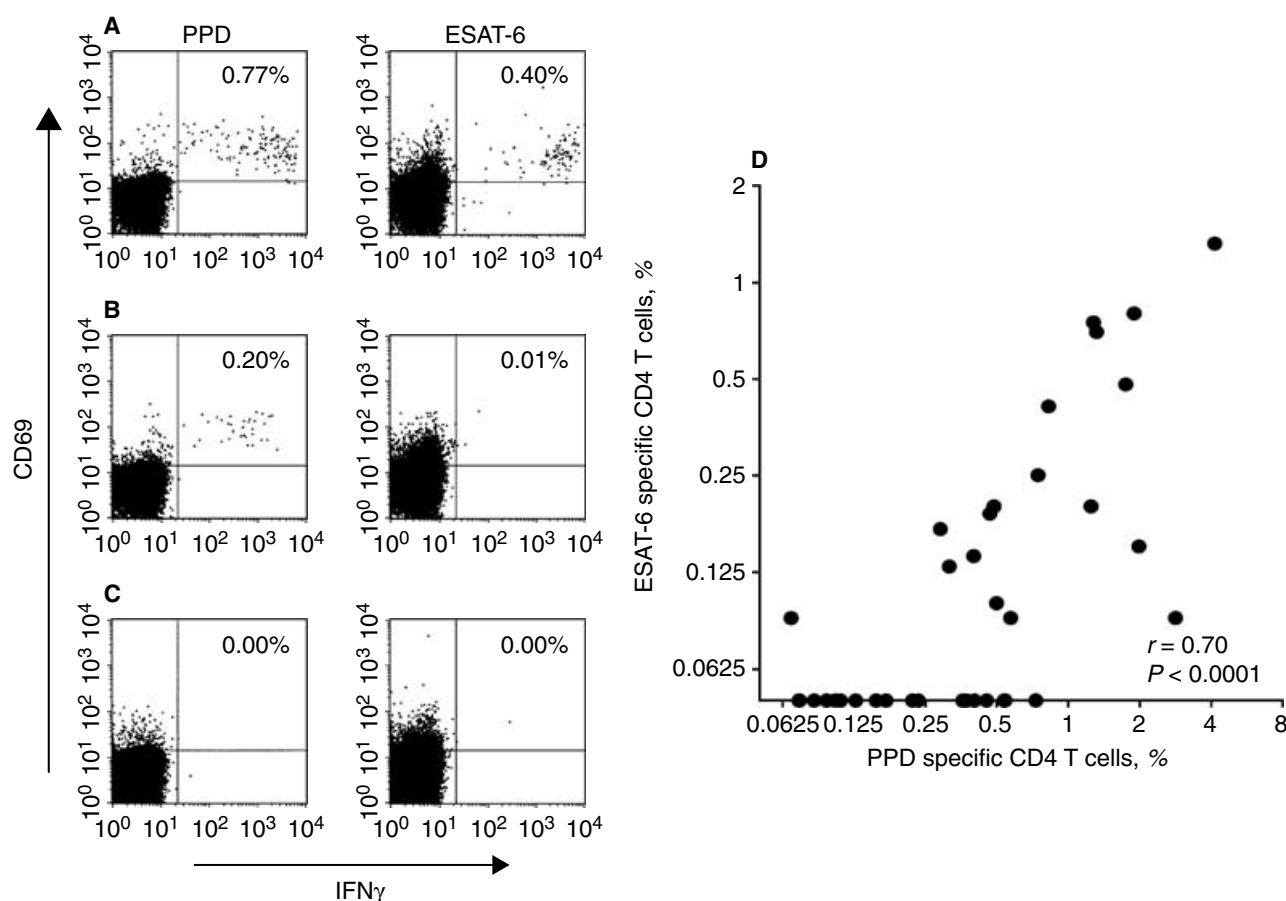
PPD is a complex mixture of proteins that may also detect T-cell reactivity toward vaccinations using the *M. bovis* BCG strain. Thus, PPD-specific T-cell reactivity in the skin test or in the flow cytometric assay may either be the result of an actual latent tuberculosis infection, and/or may reflect the response toward a previous BCG vaccination. To distinguish between these possibilities, T cells were stimulated using the recombinant ESAT-6 protein that is absent from all strains of the *M. bovis* BCG vaccine. After stimulation of whole blood with PPD and ESAT-6 antigen, differential response patterns were observed. Dotplots of the flow cytometric analysis of T cells reactive toward ESAT-6 in PPD-positive and -negative individuals are shown in Figure 5A to C. Interestingly, whereas the individual shown in Figure 5A exhibited T-cell reactivity toward both PPD (0.77%) and ESAT-6 (0.40%), the T-cell response of the individual shown in Figure 5B was restricted to PPD only. Moreover, individuals lacking a response toward PPD never showed any reactivity toward ESAT-6 (Fig. 4C, and data not shown). Among a total of 36 PPD-positive HD patients who were analyzed, 18 had detectable T-cell responses toward ESAT-6 (Fig. 4D). Similarly, among a group of 37 immunocompetent control individuals with detectable PPD-specific T-cell reactivity, 15 were also positive for ESAT-6 (data not shown). Interestingly, there was a significant correlation between the frequencies of specific CD4 T cells that were stimulated by PPD and ESAT-6 ( $r = 0.72$ ,  $P < 0.0001$ ), and the differentiation status of ESAT-6 reactive cells did not differ from PPD-specific T cells ( $55.07 \pm 24.01\%$  CD27-positive cells among ESAT-6 reactive T cells) (compare with Table 2). Taken together, although BCG vaccination status was not consistently available,

the presence of T cells reactive toward ESAT-6 is strongly indicative of a latent *M. tuberculosis* infection, and is not a result of previous BCG vaccination [19, 25, 26].

#### DISCUSSION

In this study, the prevalence of latent *M. tuberculosis* infection was characterized in healthy individuals and hemodialysis patients with uremic immunodeficiency, and the frequencies and phenotypic characteristics of PPD-specific CD4 T cells were analyzed using a rapid flow cytometric whole-blood assay. Interestingly, the prevalence of PPD reactivity was lower in immunocompetent individuals with low risk of exposure compared with the higher risk groups of both health care workers and hemodialysis patients. The latter groups, however, did not differ with respect to prevalences or frequency distributions of PPD-specific T cells. Given the well-known impaired responsiveness of hemodialysis patients in skin tests [5, 6], our findings indicate that the whole blood assay may have potential as a useful alternative to skin testing, and suggest that this assay may have an improved sensitivity for the diagnosis of a latent tuberculosis infection in immunocompromised individuals.

Interestingly, a significant correlation was found between the frequencies of PPD-reactive T cells and the size of the skin-test induration. This is conceivable, as both the skin test and the whole-blood assay rely on similar immunologic principles [27]. Both assay systems are based on the specific induction of inflammatory cytokines by T cells that have previously been sensitized to mycobacterial antigens in vivo. In the skin test, the characteristic induration is caused by the infiltration of PPD-specific lymphocytes, resulting in a local inflammatory response at the site of injection [16]. In the whole-blood assay, the induction of cytokines is used as a measure to directly quantitate PPD-reactive T cells after specific stimulation in vitro. Our data show that the sensitivity of the skin test is similar to the T cell-based test in immunocompetent



**Fig. 5. Identification of latently infected individuals using the early secretory antigenic target-6 (ESAT-6) antigen.** Representative dot plots of individuals with or without a specific T-cell reactivity toward purified protein derivative (PPD) or ESAT-6 (A-C). Whole blood was stimulated with control antigen (diluent, not shown), PPD, or ESAT-6, and specifically induced interferon  $\gamma$  (IFN $\gamma$ ) induction in CD4 T cells was analyzed using flow cytometry. Numbers indicate the percentage of specifically stimulated CD4 T cells. Specific T-cell frequencies toward ESAT-6 were analyzed in 36 PPD-positive hemodialysis patients, and 18 individuals had ESAT-6-specific T cells above 0.05% (D). T-cell frequencies toward PPD and ESAT-6 showed a significant correlation ( $r = 0.70$ ,  $P < 0.0001$ ).

individuals (Table 3). In immunocompromised individuals, however, a far lower sensitivity was observed for the skin test, as has been previously described [5, 6]. This may be a consequence of the impaired costimulatory capacity of uremic antigen-presenting cells and cellular immune reactivity in vivo [7, 8, 10, 11]. In contrast, hemodialysis patients did not show impaired PPD reactivity in vitro, suggesting that the sensitivity of this T cell-based test may be unaffected by immunosuppression. This may in part be because the in vitro assay allows optimization of stimulatory conditions to reveal a maximum amount of the individual T-cell reactivity. This is mainly achieved by the addition of costimulatory antibodies, which were shown to lead up to a 3-fold increase in the detection of actual T-cell frequencies without causing nonspecific activation [28].

The sensitive identification of latent infections may help to target preventative antibacterial prophylaxis to immunocompromised patients. This may be particularly relevant for latently infected hemodialysis patients await-

ing or undergoing transplantation, as the risk of active disease is increased 10-fold in hemodialysis patients and 50-fold in transplant recipients [29]. In the setting of transplantation, preventative prophylaxis is recommended for patients with a previous history of tuberculosis, or for patients with strong skin-test reactivity [17, 18]. In immunocompetent individuals, *M. tuberculosis*-specific immune responses are generally sufficient to maintain long-term immune control and clinical latency. Immunosuppressive treatment, however, may result in the disruption of this well-balanced equilibrium, and in the progression to symptomatic active tuberculosis. In line with this evidence, active tuberculosis is often associated with falsely negative skin tests [16] and reduced levels of PPD-specific immunity in circulation [30, 31]. This situation is reminiscent of the specific T-cell immunity against a number of persistent viruses such as cytomegalovirus (CMV), where an equilibrium between specific immunity and viral replication exists. We have recently shown in the setting of renal transplantation, that stable levels

of CMV-specific T cells correlate with an efficient control of viral replication, whereas a decrease of CMV-specific CD4 T cells is associated with the progression to symptomatic CMV disease [22]. Thus, it is tempting to speculate whether the serial analysis of PPD-specific T-cell immunity in latently infected patients may similarly be applied for the monitoring of infectious complications after transplantation.

In the past years, particular interest was focused on deleted regions in the genome of the *M. bovis* BCG strain. T-cell responses to ESAT-6 and CFP-10, antigens encoded by genes that are deleted from BCG but present in *M. tuberculosis*, can discriminate latently infected patients from individuals vaccinated with BCG or sensitized to nontuberculous mycobacteria [25–27, 32–34]. Based on stimulation with PPD alone, neither the skin test nor the in vitro flow cytometric assay allows a clear-cut distinction. Clinical observations indicate that cross-reactions derived from BCG vaccinations tend to result in smaller amounts of induration than reactions caused by *M. tuberculosis*, although overlap exists [18, 35]. Conversely, latent infections are rather associated with stronger delayed-type hypersensitivity responses. In line with this evidence, T-cell reactivity toward ESAT-6 was primarily found in individuals with high frequencies of PPD-specific T cells (Fig. 4D). This indicates that high levels of PPD reactivity are associated with positive responses toward ESAT-6, and are particularly suggestive of latent *M. tuberculosis* infection. Conversely, low levels of PPD reactivity may correlate with vaccination responses. Our data may suggest a rough estimate of the thresholds of PPD reactivity that may be indicative of an actual latent infection. In the current study, a PPD-reactivity above approximately 0.3% was associated with a positive T-cell response toward ESAT-6 in 74% of all cases (Fig. 4D). Together these data highlight how a latent infection imposes a constant challenge on the host immune system to maintain high levels of protective cellular immunity.

Similar to *M. tuberculosis*, viruses such as HIV-1, Epstein-Barr virus, CMV, or hepatitis C virus are characterized by the establishment of lifelong infection in the human host, where their replication is tightly controlled by virus-specific cellular immune responses [36–40]. Interestingly, the differentiation phenotype of antigen-specific T cells may differ considerably depending on the viral specificity, and on the infection status of the host [41–43]. During primary infections, specific T cells are generally characterized by the expression of CD27, which is indicative of a recently activated phenotype [41, 44]. Interestingly, PPD-specific T cells are memory cells containing equal amounts of both CD27-positive as well as terminally differentiated T cells (CD27-negative; Fig. 4A and Table 3). Along with the presence of stable T-cell frequencies over time, this mixed phenotype may indicate

the need for a constant supply of PPD-reactive T cells as a consequence of regular bacterial challenge.

## CONCLUSION

Taken together, the use of a rapid flow cytometric assay was evaluated for the sensitive detection of latent infection with *M. tuberculosis* in immunocompromised individuals. So far, this has not been shown for other tests that analyze T-cell function, such as proliferation assays or enzyme-linked immunosorbent assay (ELISA)-based QuantiFERON approaches [45, 46]. One other T-cell test, the enzyme-linked immunospot (ELISPOT) assay, however, also allows for an accurate quantitation of specific T cells at the single-cell level. This approach was similarly effective in detecting T-cell responses toward *M. tuberculosis* in immunocompromised individuals; unlike the skin test, it was not adversely affected by HIV infection [32]. The low threshold for detection of antigen-specific T cells in ELISPOT (0.004% of peripheral blood mononuclear cells) may explain its high diagnostic sensitivity and its widespread utility in a number of clinical settings [19, 25, 26, 32]. However, the flow cytometric approach has certain advantages, as it may be performed directly from whole blood from small sample volumes (<1 mL) without the need of cell isolation before stimulation. Apart from quantitation of antigen-specific T cells, it also enables simultaneous immunophenotyping of reactive T cells with respect to T-cell subpopulations, differentiation status, and cytokine profiles. Additionally, in vitro assays in general have a number of advantages over the use of the established skin test. First, they can be performed directly from whole blood within a single day and do not require several visits by the patient for placing and reading of the test. Second, no particular exclusion criteria exist, because the tests can be applied in patients with skin disorders or in patients with a previous history of tuberculosis infection. Third, there is no need to do serial tests with increasing doses of antigen, and tests can be repeated on separate occasions without being confounded by the booster effect on specific immunity in vivo. Finally, the potential to conveniently characterize and quantitate immune responses toward selected mycobacterial antigens or antigenic mixtures enables us to distinguish actual latent infection from purely vaccination-induced responses.

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